5-2015

The Effects of ZnO Nanoparticles on Egg, Larva, and Adult Rough-Skinned Newts (Taricha granulosa)

Austin Reid Spence
Utah State University

THE EFFECTS OF ZNO NANOPARTICLES ON EGG, LARVA, AND ADULT ROUGH-SKINNED NEWTS (*TARICHA GRANULOSA*)

by

Austin Reid Spence

Thesis submitted in partial fulfillment
of the requirements for the degree

of

HONORS IN UNIVERSITY STUDIES
WITH DEPARTMENTAL HONORS

in

Biology
in the Department of Biology

Approved:

Dr. Susannah French	Dr. Kim Sullivan

Dr. Kristine Miller

UTAH STATE UNIVERSITY
Logan, UT

Spring 2015

Acknowledgements

I would like to thank Dr. Susannah French for her continual support and mentoring throughout my undergraduate career at Utah State University. I would also like to thank Dr. Edmund Brodie, Jr. and Gareth Hopkins for guidance on completing this project. I would like to thank doctoral candidates Geoff Smith and Lori Neuman-Lee for being fantastic mentors and helping me figure out how to be a better scientist, presenter, and future graduate student. Finally, I'd like to thank Marilize Van der Walt for data collection and being a great lab-mate.

Table of Contents

Preface

Manuscript

Honors Material

List of Figures

Abstract:

The objective of this study was to examine the effects of zinc oxide (ZnO) nanoparticles on egg, larva, and adult rough-skinned newts, *Taricha granulosa*. To date, little research has been done to investigate the potentially detrimental effects of nanoparticles on amphibians, especially salamanders and newts (caudates). Chronic toxicity was tested on eggs and larvae, and acute toxicity was tested on eggs, larvae, and adults. For eggs, chronic exposure to ZnO nanoparticles caused higher mortality at 10.0 and 100.0 mg/L compared to 0.0, 0.1, and 1.0 mg/L. When given an acute exposure (24h) to nanoparticles at a late incubation stage, nanoparticles caused larvae to hatch five days early, at a decreased developmental stage and smaller size compared to the control. Chronic and acute exposure of larvae increased mortality up to 75% at both 10 and 100.0 mg/L, and exhibited sublethal effects, most dramatically, severe gill degradation. These results suggest nanoparticles can have lethal and sublethal effects on all life stages of amphibians. For the first time to our knowledge caudates are shown to be vulnerable to nanoparticle toxicity. Considering the paucity of data on this relatively new environmental contaminant, the use of nanoparticles and their potential effects need to be recognized as these substances become increasingly prevalent in the environment.

1. Introduction:

Metal oxide nanoparticles, a unique and new type of pollutant, are increasing in usage (Oberdörster et al. 2007) as nanoparticles are now being used in products that range from cosmetics to pesticides (Panácek et al. 2009; Liu et al. 2008; Mu et al. 2010). Nanoparticles are defined as particles less than 100 nanometers in length (SCENIHR 2006). Due to their large surface area:mass ratios, nanoparticles have many applications in chemistry, agriculture, medicine, and material science (Daniel et al. 2004; Dimpka et al. 2012; Salata et al. 2004; Chol et al. 1995). These qualities raise new questions in toxicology because compounds smaller than 100 nanometers have been shown to be more toxic than the same compound in bulk form (Borm 2002) due to increased surface area.

Aquatic environments often act as sinks for environmental contaminants, and current research suggests nanoparticles act in a similar way to other colloid pollutants in aquatic environments (Petosa et al. 2010; Moore 2006). It is imperative to understand the effects of nanoparticles on the organisms that will come into contact with them in the environment. Current research shows that nanoparticles can have deleterious effects on aquatic organisms, including fish (Zhu et al. 2008), macroinvertebrates (Lovern et al. 2006), and algae (Navarro et al. 2008). Current evidence as to the effects of nanoparticles on amphibians is limited to two studies on frogs (Nations et al. 2011; Nations et al. 2011). To our knowledge, nothing is known about the effects on caudate amphibians (salamanders and newts) or across multiple life-history stages of any one species. Studies on amphibians are particularly critical at this time, as rapid amphibian declines have been observed since the 1950s (Houlahan et al. 2000; Alford et al. 2001; Sodhi et al. 2008;

Wake and Vrendenburg 2008), and among the many causes, such as habitat destruction or disease (Stuart et al. 2005; Daszak et al. 1999; Collins et al. 2004), pollution is particularly apparent (Blaustein et al. 2003; Collins et al. 2003; Hayes et al. 2002; Davidson 2004).

This experiment examines the effects of ZnO nanoparticles on the mortality of eggs, larvae, and adults of the rough-skinned newt (*Taricha granulosa*). This is the first study on a caudate amphibian as well as the first study to test the effects of nanoparticles at multiple life history stages. Mortality, growth, and development were measured in eggs and larvae, but adults allowed for a wider spectrum of analyses, including corticosterone, immunocompetence ability, and oxidative stress. Corticosterone (CORT) is an energy-mobilizing glucocorticoid that is often used as an indicator of stress (Sapolsky 1992). This alone does not provide a comprehensive representation of an organism's health, however, and therefore immunocompetence and oxidative stress were also measured as critical components of an organism's health (Lochmiller et al. 2000). Using these data, we addressed the following questions: (1) How do ZnO nanoparticles affect embryos and larvae of newts exposed chronically at multiple concentration levels? (2) How do ZnO nanoparticles affect embryonic and larval newts exposed acutely at different developmental periods? and (3) How do ZnO nanoparticles affect adult newts exposed acutely? We hypothesized ZnO nanoparticles would adversely affect newts at all life history stages, with chronic exposure to ZnO nanoparticles causing higher mortality than acute exposure, negative effects on development and size, and more deformities relative to controls.

2. Methods and Materials:

2.1 Experimental Animals:

Ten gravid adult female rough-skinned newts (*Taricha granulosa*) were collected from Hunter Creek, Curry County, Oregon (42°23'19.60" N, 124°25'21.54"W) in May 2014. At Utah State University, they were housed individually in plastic containers with 2.0 L of filtered dechlorinated tap water and a styrofoam perch in an environmental controlled chamber at 14° C on a 12-h: 12-h light/dark cycle.

After 48 h acclimation, females were injected with 10 µL luteinizing-hormone-releasing-hormone ([des-Gly10,D=His(Bzl)6]-LHRH ethylamide; Sigma #L2761) to induce oviposition onto provided polyester fiber. All eggs were deposited between May 20 and June 10, 2014 and were collected and separated from the oviposition site within 24 h. Newt eggs were reared based on a previous protocol presented in Hopkins et al. (2012) with the exception that eggs were reared singly in Petri dishes, not in groups of three.

2.2 Solution preparation:

Treatment solutions included 0.0 mg/L (filtered dechlorinated water, hereafter control), 0.1 mg/L, 1.0 mg/L, 10.0 mg/L, and 100.0 mg/L of ZnO nanoparticles (Sigma Alridch, #721077), made by mixing ZnO nanoparticles with control solution. Nanoparticle concentrations were selected from previous work on tadpoles (Nations et al., 2011). All solutions were stored in sealed glass jars at 14° C in the same environmental chamber as the newts. Solutions were not sonicated but rather mixed before being dispensed to simulate a more natural environmental setting, similar to other experiments (Nations et al.

2011). Once in Petri dishes, test solutions were not stirred, as this could be stressful to the animal. ZnO nanoparticles precipitated out of solution at 100.0 mg/L, but this was acceptable because if they precipitate in the lab they will most likely precipitate in the environment as well.

Two types of experiments were performed: chronic exposure and acute exposure. Chronic exposure experiments were completed with eggs and larvae, while acute exposure experiments were completed with eggs, larvae and adults, resulting in six total experiments.

2.3 Experiment 1: Chronic Exposure of Eggs:

Eggs (N=537) were randomized to treatment groups: control, 0.1 mg/L, 1.0 mg/L, 10.0 mg/L, or 100.0 mg/L of ZnO nanoparticles. Four mL of solution was pipetted into a 3.5 cm diameter, 1 cm deep, round, plastic Petri dish (hereafter, Petri dish), into which one randomly assigned egg was placed. Newt eggs were checked daily for mortality or hatching. Length at hatching was measured using an ocular micrometer on an Olympus stereomicroscope, and developmental stage at hatching was determined using the salamander early life-history staging protocol of Harrison (1969). Larvae were kept in Petri-dishes for two weeks after hatching, and were re-measured and staged at this point to determine growth and development.

2.4 Experiment 2: Acute Exposure of Eggs:

Eggs (N=400) were reared in control solution. Eggs were exposed to treatments of either control or 10.0 mg/L ZnO nanoparticles for 24 h at 1, 9, 17, or 25 d after oviposition. Four mL of solution was pipetted into a new Petri dish, into which one randomly assigned egg was placed. After 24 h exposure, the eggs were returned to a Petri dish of control solution. Eggs were checked daily for hatching and mortality and hatched larvae were checked daily for mortality. Length and developmental stage were measured at hatching and two weeks after hatching, as before.

2.5 Experiment 3: Chronic Exposure of Larvae:

Eggs (N=240) were reared in control solution. Eggs were checked daily for hatching. Length and developmental stage were measured at hatching. Hatched larvae were randomly assigned to one of five of the following treatment groups: control, 0.1 mg/L, 1.0 mg/L, 10.0 mg/L, or 100.0 mg/L of ZnO nanoparticles, and were placed singly in 4 mL of treatment solution in a new Petri dish immediately upon hatching. Larvae were checked daily for mortality. Length and developmental stage were measured again two weeks after hatching, at which point the experiment ended.

2.6 Experiment 4: Acute Exposure of Larvae:

Eggs (N=400) were reared in control solution. Eggs were checked daily for hatching. Length and developmental stage were measured upon hatching. Larvae were exposed to treatments of control or 10.0 mg/L ZnO nanoparticles for 24 h at 1, 4, 7, or 10 d after hatching. At the treatment day, 4 mL of treatment solution was pipetted into a Petri dish, into which one randomly assigned larva was placed. After 24 h, larvae were returned to a

Petri dish of control solution. Eggs were checked daily for hatching and mortality. Larvae were checked daily for mortality. Length and developmental stage were measured at hatching and two weeks after hatching, as previously described in Section 2.5.

2.7 Experiment 5: Chronic Exposure of Eggs and Larvae

Eggs (N=91) were randomized to treatment groups: control, 0.1 mg/L, 1.0 mg/L, 10.0 mg/L, or 100.0 mg/L of ZnO nanoparticles. Eggs were checked daily for hatching, upon which length and developmental stage were measured. Larvae were then placed into control solution or fresh corresponding treatment solution. Larvae were checked daily for mortality. Length and developmental stage were measured at hatching and two weeks after hatching, as previously described in Section 2.5.

2.8 Experiment 6: Acute Exposure of Adults

Adult newts (N=36) were housed in individual plastic containers of control solution at 14° C with 12-h :12-h light/dark cycle. Newts were randomly assigned a treatment group, 0.0 or 100.0 mg/L ZnO nanoparticle solution, and exposed to 500 mL of 100.0 mg/L ZnO nanoparticle solution for 24 h. At 24 h, newt tail tips were removed and blood was collected from the caudal vein using a capillary tube. Blood was centrifuged and the plasma was collected and frozen at -80°C until ready to be assayed.

2.9 Radioimmunoassays, bacterial killing assays, and oxidative stress assays

CORT concentrations were determined using a radioimmunoassay protocol adapted from French et al. (2008). In brief, samples were extracted using a solution of 30% ethyl

acetate: isooctane in duplicate for CORT (MP Biomedicals, Lot #3R3PB-19E). Final concentrations were adjusted using individual recoveries. Microbiocidal assays were performed following French and Neuman-Lee (2012). In brief, 1:5 plasma dilution with CO_2-independent media and L-glutamine were combined with 10^4 colony-producing units of *Escherichia coli* (EPowerTM Microorganisms #483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, MN) and agar broth. Samples were incubated in a 96-well microplate for 12 h and absorbance was calculated using a microplate reader (300nm, BioRad Benchmark, Hercules, CA). Reactive oxygen metabolites (ROMs) in the plasma were measured to determine if there were elevated levels of oxidative stress. We followed the protocol included with the d-ROMs Test kit (Diacron, Grosseto, Italy). Briefly, we mixed the provided R1 and R2 reagents in a 1:100 dilution to create an acidic buffered solution with a chromogen. This resulting solution was kept in the dark until 5 µl of sample plasma was added into separate wells of a 96-well microplate and 100 µl of the R1/R2 solution was added to each well. Nanopure water was used as sample blanks and the provided serum was used as a calibrator solution. We followed the "end-point mode" from the manufacturer's protocol and measured absorbance at 505 nm after a 90 m incubation at 37° C. The resulting units are in mg H2O2/dl (1 CARR U = 0.08 mg H2O2/dl).

2.10 Statistical analyses

Mixed effects models were used to predict the mortality rate of eggs and larvae at different nanoparticle concentrations for chronic experiments. Data were analyzed in the program R 3.1.1 with the packages "nlme", "arm", "lme4", and "stats" (Pinheiro et al.

2015; Bates et al. 2014; Gelman et al. 2014; R Core Team 2014) with mixed effect models. The full model for egg and larval experiments is as follows:

$$\Pr(\text{Death}_{ij}=1) = \text{logit}^{-1}(\alpha_{ij} + \beta_i(\log(\text{concentration}))_i)$$

$$\alpha_j \sim N(\mu_\alpha, \sigma^2_\alpha) \text{ for } j=1,2,\ldots j \text{ and } j=\text{female}$$

The probability of death is given by a logistic function with an intercept of α and slope of β. The random effect of female affected the intercept. The concentration of nanoparticles was centered for the model.

Sublethal continuous variables, including size, developmental stage, and days to hatch, were analyzed in R using ANOVA's. For chronic experiments, data were compared by concentration of ZnO nanoparticles. For acute experiments, data were compared by treatment to control within treatment day. Adult corticosterone data were analyzed separately by sex due to different responses to treatment. No differences were seen between the sexes for bacterial killing ability ($p = 0.0545$) and oxidative stress ($p = 0.371$), so sexes were combined to give a larger sample size.

3. Results:

Egg and larval mortality significantly increased as the concentration of ZnO nanoparticles increased for both chronic egg and larval experiments. Nanoparticles affected larval mortality more than egg mortality. Treated adult females showed higher corticosterone levels.

3.1 Experiment 1: Chronic Exposure to Eggs:

Egg mortality significantly increased as the concentration of ZnO nanoparticles increased (Fig 1). A final mixed effects model is given by:

$$\Pr(\text{Death}_{ij}=1) = \text{logit}^{-1}(-2.309 + 0.6294 \log(\text{concentration})_i)$$

$$\alpha_j \sim N(0, 1.169^2_\alpha) \text{ for } j=1,2,\ldots j \text{ and } j=\text{female}$$

The effect of nanoparticle concentration was highly significant ($p < 0.001$) and female was used as a random effect to account for variation.

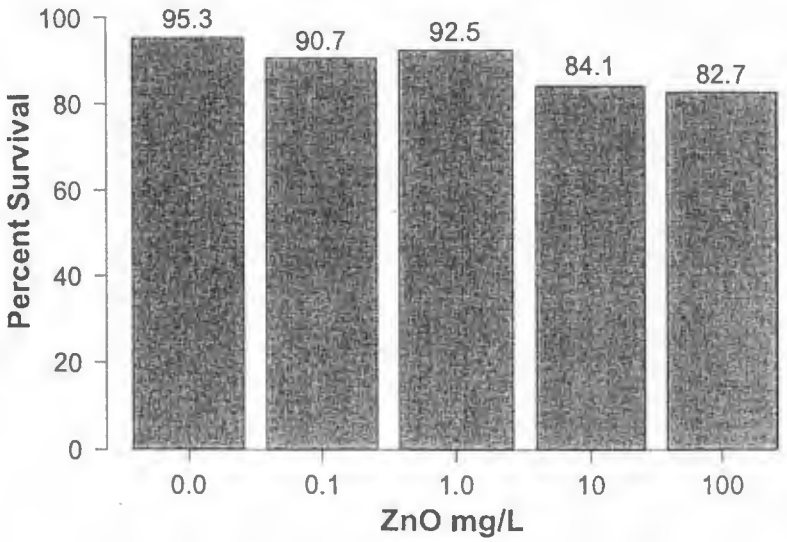

Fig 1 Percent survival of chronically exposed eggs to different concentrations of ZnO nanoparticles.

It appears that nanoparticles may have degraded the egg layer, causing the eggs to open and forcing larvae to hatch earlier. Comparison of a control egg and treated egg in 100.0 mg/L ZnO show a white substance and degraded egg layer on the treated egg (Fig 2).

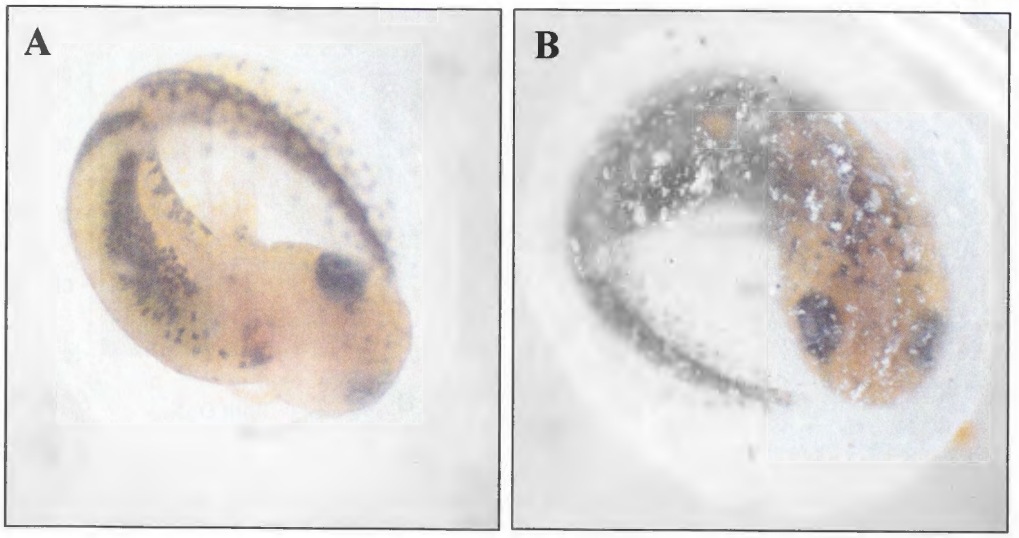

Fig 2A (left) shows a newt egg in control solution with healthy egg layers. **2B** shows an egg treated with 100.0 mg/L ZnO nanoparticles. There is a white substance and visible degradation on the outer egg layer

3.2 Experiment 2: Acute Exposure to Eggs:

Egg mortality did not increase when exposed to 10.0 mg/L ZnO nanoparticles for 24 h when compared to the control. Eggs treated on day 1, 9, or 17 showed no difference in size, developmental stage, time to hatch, or mortality when compared to the control. Eggs treated on day 25 hatched 5 d earlier on average ($F_{1,96}$=53.96, $p<0.001$) than the control. When hatched, larvae were significantly shorter ($F_{1,96}$=39.69, $p<0.001$) and less developed ($F_{1,96}$=67.42, $p<0.001$) (Fig 3).

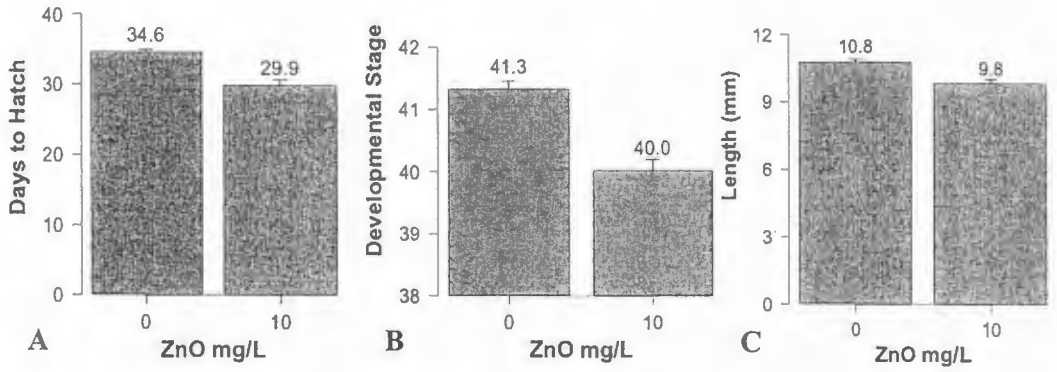

Fig 3A (left) shows eggs exposed at day 25 to zinc oxide hatched significantly earlier. **3B** (middle) shows when larvae hatched less developed when eggs were treated at day 25, and **3C** (right) shows treated larvae hatched smaller when eggs were treated at day 25.

3.3 Experiment 3: Chronic Exposure to Larvae:

Larval survival was significantly effected by ZnO nanoparticle concentration, with significantly greater mortality at 10 and 100 mg/L than at control, 0.1, or 1.0 mg/L (Fig 4). A final mixed effects model is given by:

$$\Pr(\text{Death}_{ij}=1) = \text{logit}^{-1}(-1.303 + 2.7192 \log(\text{concentration})_i)$$

$$\alpha_j \sim N(0, 0.2635^2{}_\alpha) \text{ for } j=1,2,\ldots j \text{ and } j=\text{female}$$

The effect of nanoparticle concentration was highly significant ($p < 0.001$) and female was used as a random effect to account for variation.

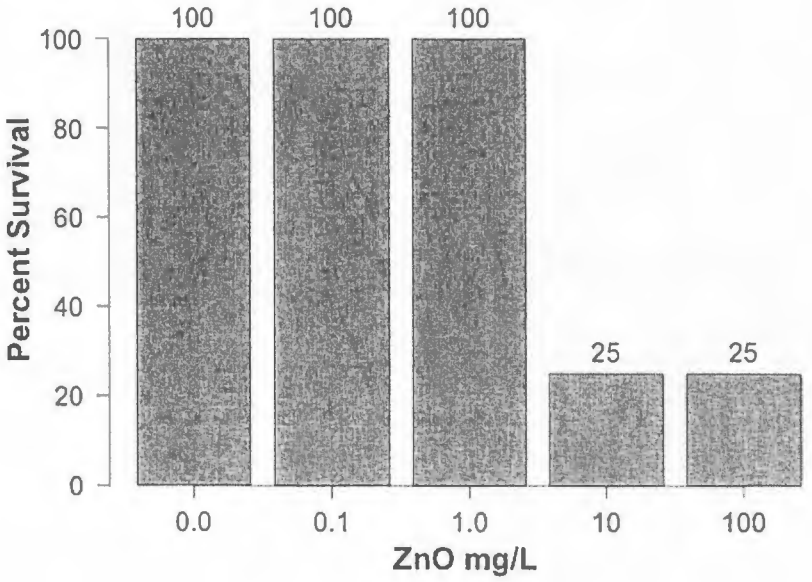

Fig 4 Percent survival of chronically exposed larvae to different concentrations of ZnO nanoparticles

Seventy-five percent of newt larvae exposed to 10 or 100.0 mg/L ZnO nanoparticle concentration also exhibited severe gill degradation. Gill degradation occurred within 24-72 h of exposure and reduced the gills from long and filamentous to short and near the body, essentially destroying the gills completely (Fig 5).

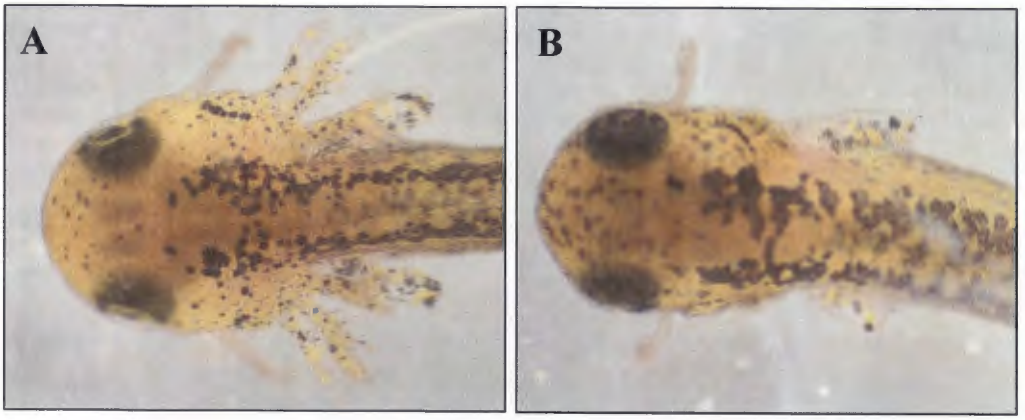

Fig 5A (left) shows a newly hatched newt larva newt larvae with healthy gills. **5B** (right) shows a larva after 1 day of treatment in 100.0 mg/L ZnO nanoparticles

3.4 Experiment 4: Acute Exposure to Larvae:

No larvae died in the control groups at any day treated. Larval mortality was significantly greater than the control when exposed to ZnO nanoparticles for 24 h at all time periods, with increasing mortality at later time periods (Fig. 6). Between 74 and 84% of larvae exposed to 10.0 mg/L ZnO nanoparticle concentration exhibited gill degradation (Fig 5) within 24 h of exposure.

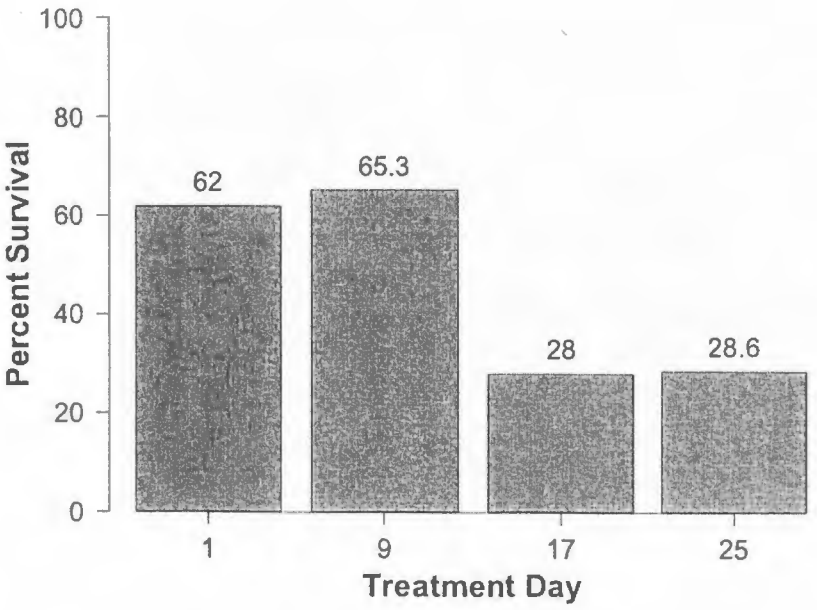

Fig 6 Percent survival for the 10.0 mg/L treatment group at all treatment days. The control group at each treatment day is not shown because all control groups had 0% mortality.

3.5 Experiment 5: Chronic Exposure of Eggs and Larvae

All larvae raised at control solution survived, regardless of the solution the eggs were raised in. Larvae that were reared embryonically in ZnO nanoparticles surviving fine in this solution upon hatching, but died if transferred to a new Petri dish with 10 or 100 mg/L ZnO solution (Fig 7). These larvae that died in new 10 and 100 mg/L also showed degraded gills.

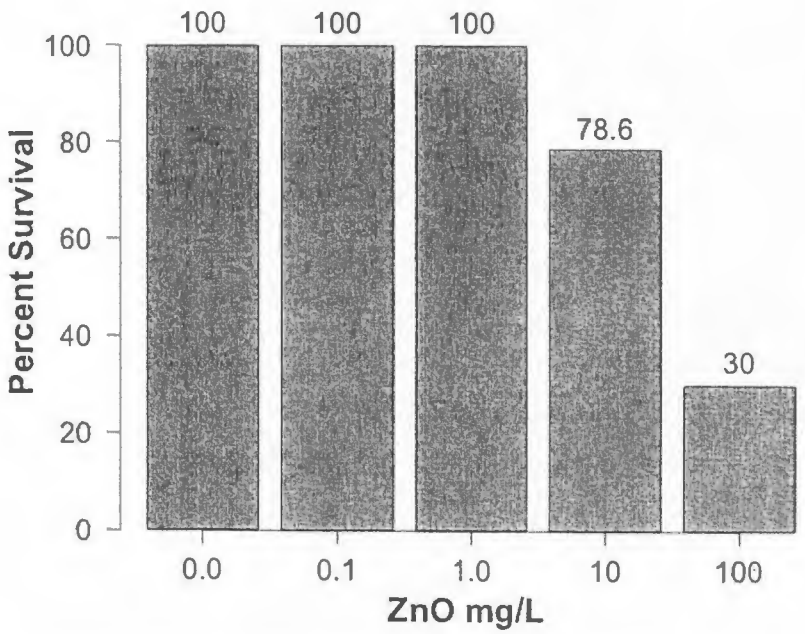

Fig 7 Percent survival for larvae transferred to new ZnO concentrations upon hatching. All larvae in control solution survived, regardless of what concentration the egg was raised in.

3.6 Experiment 6: Acute Exposure of Adults

Adult female newts had significantly higher corticosterone levels ($F_{1,16} = 5.053$, $p = 0.039$), and males were almost significantly greater ($F_{1,16} = 4.086$, $p = 0.0603$) (Fig 8). Neither males nor females showed differences in oxidative stress ($F_{1,32} = 0.05$, $p = 0.824$) or bacterial killing ability ($F_{1,34} = 0$, $p = 0.994$) among treatments.

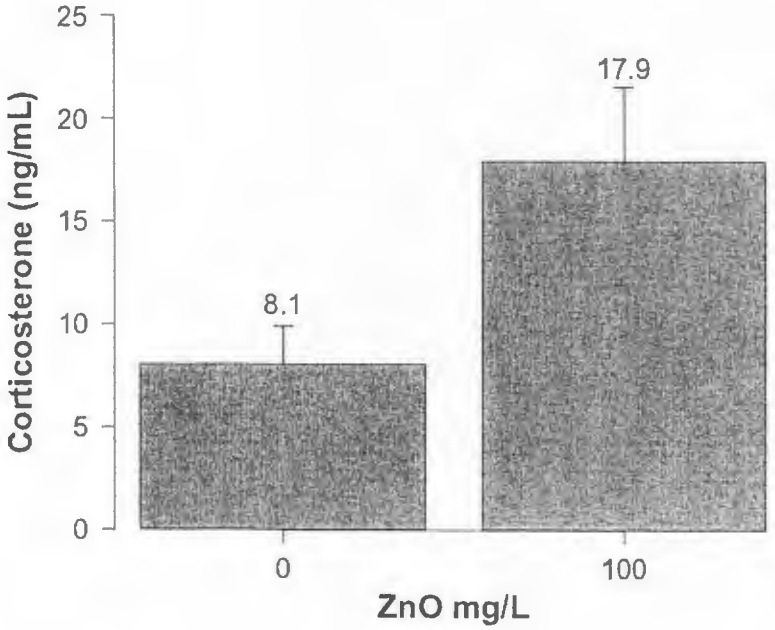

Fig 8 Corticosterone levels of females in both the control and 100 mg/L ZnO nanoparticle solution.

4. Discussion:

Our results demonstrate that ZnO nanoparticles had significant negative effects on all life stages of newts. Chronic exposure of eggs dramatically increased mortality. Acute exposure of eggs decreased time to hatch and subsequent size and developmental stage at hatching. Chronic and acute exposure to larvae significantly increased mortality and caused severe gill degradation (Fig 4). When exposed to new solution, larvae exposed at 10 and 100 mg/L experienced mortality while all larvae exposed to control solution survived. Acute exposure to adult newts increased female corticosterone (physiological stress). Our results support previous studies showing that ZnO nanoparticles increase mortality and have serious sublethal effects on aquatic organisms, including crustaceans, zebrafish, bacteria, and frogs (Zhu et al. 2008; Heinlaan et al. 2008; Tollefsen et al. 2008; Nations et al. 2011).

Chronic exposure of ZnO nanoparticles to eggs increased mortality. Although no other studies on amphibian eggs exist, nanoparticles, including ZnO, have also been shown to cause mortality in zebrafish embryos (Zhu et al. 2008; Bar-Ilan et al. 2009). Acute exposure did not affect eggs until the last quarter of their development. When exposed, eggs hatched 5 d earlier causing them to hatch less developed and smaller. These sublethal effects can significantly affect survival to adulthood because small, less developed larvae are more prone to the negative effects of pollution (Hopkins et al. 2014; Beebee 1986; Cooke 1979), higher predation and competition, as well as learning feeding behaviors slower (Warkentin 1995; Warkentin 1999; Boone et al. 2002; Gall et al. 2011). Salamanders have been shown to be phenotypically plastic in their hatch time, and can

sense both predators and chemicals given off by predators (Sih et al. 2003). Being able to alter timing of hatching allows larvae to stay in the egg shorter or longer depending on the current environment of the egg. If nanoparticles are creating a negative environment, larvae may be hatching earlier to become mobile and get to healthier surroundings.

Chronic exposure of larvae significantly increased mortality at 10 and 100.0 mg/L. Larvae exposed to these levels of ZnO nanoparticles also showed severe degradation of gills. Acute exposure to larvae showed significantly higher mortality at all developmental stages, but mortality increased the older and larger the larva was. Gill degradation was also seen in larvae treated for only 24 h. Overall, chronic exposure of ZnO nanoparticles affected mortality more significantly than acute exposure. Similar results of longer exposure times increasing mortality were seen in a previous study testing zinc pollution on *X. laevis* (Haywood et al. 2004). A different possibility may be ZnO nanoparticles turning into soluble zinc (Zn). Several studies have evaluated ZnO nanoparticles at the same time as soluble Zn. ZnO nanoparticles showed similar toxicity to soluble Zn, and the observed toxicity may simply be due to soluble Zn (Mortimer et l. 2008; Franklin et al. 2007; Heinlaan et al. 2008; Aruoja et al. 2009) due to the ability of ZnO to create soluble Zn in the water column (USEPA 2005). Larval mortality of newts is correlated with gill destruction and may be due to nanoparticles possibly having an affinity for ciliated or filamentous ectoderm cells (Nations et al. 2010). Nanoparticles have been previously shown to adhere to those portions of tadpoles based on scanning electron microscopy of *Xenopis laevis* skin. This would explain why older larvae were more susceptible to 24 h of treatment. Older larvae have larger, more filamentous gills because

they have a larger metabolic need (Feder 1977). If gill are destroyed, the oxygen availability for the larvae is greatly reduced.

Eggs appear to be providing protection to larvae by causing a precipitate to form on the eggs (Fig 2B) and removing ZnO nanoparticles from solution. This is seen by the combination of experiments one, three, and five. All larvae survived in their original solution in experiment one after hatching after the eggs were in the solution for over one month, yet when larvae were exposed to 10 and 100 mg/L ZnO in experiment 3, they showed high mortality. This was corroborated by experiment 5 in which eggs exposed to ZnO solutions were also exposed to new ZnO solutions. Larvae experienced mortality at 10 and 100 mg/L, showing that the larvae were still susceptible to ZnO nanoparticles even if they experienced them as eggs, and that the eggs were taking ZnO nanoparticles out of solution.

When exposed to 100.0 mg/L ZnO solution, adult female newts showed significantly higher CORT concentrations and males showed a trend for higher CORT. These data follow previous research showing pollutants can cause increased CORT (Hopkins et al. 1997; Song et al. 2001; Hayes et al. 2006). Physiologically, this is important because an organism has a finite amount of energy and individuals must maintain a dynamic equilibrium where energy consumption is equal to energy expenditure (McCue, 2010). When stressors, such as nanoparticles, are present, more energy is needed than is available (Wingfield, 2005). These often result in changes in resource allocation between competing physiological processes (French et al., 2007; Lucas et al., 2012), i.e. self-

maintenance, immunocompetence, and reproduction. Previous research has shown high levels of CORT suppress the reproductive and immune systems (French et al. 2007; Berger et al. 2005). If our data follow this pattern, nanoparticles may have long-term effects on adult newt fitness. Although adult newts did not show additional oxidative stress when exposed to nanoparticles, previous research has shown the opposite in different organisms (Xiong et al. 2011; Huang et al. 2010). This may be due to only sampling the blood once after 24 h rather than sampling multiple times after exposure.

In conclusion, this is the first comprehensive study to examine the effects of ZnO nanoparticles on all life history stages of an amphibian. As nanoparticles become increasingly common, it is important to understand the full effects on aquatic organisms, including the mechanism behind toxicity seen in newts and other organisms. Conservation efforts for amphibians and other aquatic organisms should regard nanoparticles as an emerging and potentially dangerous new pollutant.

5. Works cited:

Alford, R.A., Dixon, P.M., et al., 2001. Global amphibian population declines. Nature 412 (6846), 499.

Aruoja, V., Dubourguier, H.C., Kasemets, K., Kahru, A., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae Pseudokirchneriella subcapitata. Sci. Total Environ. 407 (4), 1461–1468.

Bar-Ilan, Ofek, et al. "Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos." *Small* 5.16 (2009): 1897-1910.

Bates D, Maechler M, Bolker B and Walker S (2014). _lme4: Linear mixed-effects models using Eigen and S4_. R package version 1.1-7, <URL: http://CRAN.Rproject.org/package=lme4>.

Beebee, T.J.C. 1986. Acid tolerance of natterjack toad (Bufo calamita) development. Herpetological Journal 1: 78-81.

Berger, James O., and Luis R. Pericchi. "The intrinsic Bayes factor for model selection and prediction." *Journal of the American Statistical Association* 91.433 (1996): 109-122.

Berger, Silke, et al. "Corticosterone suppresses immune activity in territorial Galapagos marine iguanas during reproduction." *Hormones and Behavior* 47.4 (2005): 419-429.

Blaustein, Andrew R., et al. "Ultraviolet radiation, toxic chemicals and amphibian population declines." *Diversity and distributions* 9.2 (2003): 123-140.

Boone, M. D., D. E. Scott, and P. H. Niewiarowski 2002. Effects of hatching time for larval Ambystomatid salamanders. Copeia 2002:511–517.

Borm, A, 2002. Particle Toxicology: From Coal Mining to Nanotechnology. *Inhalation Toxicol.* 14 (3), 311–324.

Collins, J P., et al. "A model host-pathogen system for studying infectious disease dynamics in amphibians: tiger salamanders (Ambystoma tigrinum) and Ambystoma tigrinum virus." *Herpetological Journal* 14 (2004): 195-200.

Collins, James P., and Andrew Storfer. "Global amphibian declines: sorting the hypotheses." *Diversity and distributions* 9.2 (2003): 89-98.

Chol, S. U. S. "Enhancing thermal conductivity of fluids with nanoparticles."*ASME-Publications-Fed* 231 (1995): 99-106.

Cooke, A.S. 1979. The influence of rearing density on the subsequent response to DDT dosing for tadpoles of the frog Rana temporania. Bulletin of Environmental Contamination and Toxicology 21: 837-841.

Daniel, Marie-Christine, and Didier Astruc. "Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology." *Chemical reviews* 104.1 (2004): 293-346.

Daszak, Peter, et al. "Emerging infectious diseases and amphibian population declines." *Emerging infectious diseases* 5.6 (1999): 735.

Davidson, Carlos. "Declining downwind: amphibian population declines in California and historical pesticide use." *Ecological Applications* 14.6 (2004): 1892-1902.

Dimkpa, Christian O., et al. "Bioactivity and biomodification of Ag, ZnO, and CuO nanoparticles with relevance to plant performance in agriculture." *Industrial Biotechnology* 8.6 (2012): 344-357.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E., Casey, P.S., 2007. Comparative Toxicity of Nanoparticulate ZnO, Bulk ZnO, and ZnCl2 to a Freshwater Microalga (Pseudokirchneriella subcapitata): The Importance of Particle Solubility. Environ. Sci. Technol. 41, 8484–8490.

Feder, Martin E. "Oxygen consumption and activity in salamanders: effect of body size and lunglessness." *Journal of Experimental Zoology* 202.3 (1977): 403-413.

French, S.S., McLemore, R., Vernon, B., Johnston, G.I.H. & Moore, M.C. (2007) Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: Long-term corticosterone manipulations via injectable gelling material. *Journal of Experimental Biology,* **210,** 2859-2865.

Gall, B.G., Stokes, A.N., French, S.S., Schlepphortst, E.A., Brodie III, E.D., and Brodie, Jr., E.D. 2011. Tetrodotoxin levels in larval and metamorphosed newts (Taricha granulosa) and palatability to predatory dragonflies. Toxicon 57: 978-983.

Gelman, Andrew and Yu-Sung Su (2014). arm: Data Analysis Using Regression and Multilevel/Hierarchical Models. R package version 1.7-07, <URLhttp://CRAN.R-project.org/package=arm>.

Hayes, Tyrone B., et al. "Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses." *Proceedings of the National Academy of Sciences* 99.8 (2002): 5476-5480.

Hayes, Tyrone B., et al. "Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact?." *Environmental Health Perspectives* 114 (2006): 40.

Haywood, L.K., Alexander, G.J., Byrne, M.J., Cukrowska, E., 2004. Xenopus laevis embryos and tadpoles as models for testing for pollution by zinc, copper, lead, and cadmium. Afr. Zool. 39 (2), 163–174.

Heinlaan, Margit, et al. "Toxicity of nanosized and bulk ZnO, CuO and TiO 2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus." *Chemosphere* 71.7 (2008): 1308-1316.

Hopkins, G.R., Brodie, Jr., E.D., and S.S. French. 2014. Developmental and Evolutionary History affect survival in stressful environments. PLoS One 9:e95174.

Hopkins, G.R., Gall, B.G., French, S.S., Brodie Jr., E.D., 2012. Interfamily variation in amphibian early life-history traits: raw material for natural selection? Ecology and Evolution 2, 1637-1643.

Hopkins, William A., Mary T. Mendonça, and Justin D. Congdon. "Increased circulating levels of testosterone and corticosterone in southern toads, Bufo terrestris, exposed to coal combustion waste." *General and comparative endocrinology* 108.2 (1997): 237-246.

Houlahan, J.E., Findlay, C.S., Schmidt, B.R., Meyer, A.H., Kuzmin, S.L., 2000. Quantitative evidence for global amphibian population declines. Nature 404 (6779), 752.

Huang, Chuan-Chin, et al. "Oxidative stress, calcium homeostasis, and altered gene expression in human lung epithelial cells exposed to ZnO nanoparticles."*Toxicology in vitro* 24.1 (2010): 45-55.

Liu, Y et. al (2008). Stabilized polymeric nanoparticles for controlled and efficient release of bifenthrin. *Pest Management Science*, *812* (December 2007), 808–812.

Lochmiller, R.L. & Deerenberg, C. (2000) Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos,* **88,** 87-98.

Lovern, SB et. al (2006). *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles. *Environmental Toxicology and Chemistry*, *25*(4), 1132–1137.

Lucas, LeiLani D., and Susannah S. French. "Stress-induced tradeoffs in a free-living lizard across a variable landscape: consequences for individuals and populations." *PloS one* 7.11 (2012): e49895.

McCue, M.D. (2010) Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **156,** 1-18.

Moore, M. N. "Do nanoparticles present ecotoxicological risks for the health of the aquatic environment?." *Environment International* 32.8 (2006): 967-976.

Mortimer, M., Kasemets, K., Heinlaan, M., Kurvet, I., Kahru, A., 2008. High throughput kinetic Vibrio fisheri bioluminescence inhibition assay for study of toxic effects of nanoparticles. Toxicol. in Vitro 22, 1412–1417.

Mu, L et. al (2010). Application of nanotechnology in cosmetics. *Pharmaceutical Research*, *27*(8), 1746–9.

Nations, S et. al (2011). Acute effects of FeO_3, TiO_2, ZnO and CuO nanomaterials on *Xenopus laevis*. *Chemosphere*, *83*(8), 1053–61.

Nations, S et. al (2011). Effects of ZnO nanomaterials on *Xenopus laevis* growth and development. *Ecotoxicology and Environmental Safety*, *74*(2), 203–10.

Navarro, E et. al (2008). Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology (London, England)*, *17*(5), 372–86.

Oberdörster, G et. al (2007). Toxicology of nanoparticles: a historical perspective. *Nanotoxicology* 1, 2–25.

Panácek, A et. al (2009). Antifungal activity of silver nanoparticles against *Candida* spp. *Biomaterials*, *30*(31), 6333–40.

Petosa, Adamo R., et al. "Aggregation and deposition of engineered nanomaterials in aquatic environments: role of physicochemical interactions."*Environmental science & technology* 44.17 (2010): 6532-6549.

Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team (2015). _nlme: Linear and Nonlinear Mixed Effects Models_. R package version 3.1-119, <URL:http://CRAN.R-project.org/package=nlme>.

Salata, Oleg V. "Applications of nanoparticles in biology and medicine." *Journal of nanobiotechnology* 2.1 (2004): 3.

Saplosky, R. M. (1992). Neuroendocrinology of the stress response. In *Behavioral Endocrinology*, eds. J. B. Becker S. M. Breedlove and D. Crews), pp. 287-324. Cambridge, MA and London: MIT Press.

Sci. Comm. Emerg. New. Identified Health Risks (SCENIHR). 2006. The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies, Eur. Comm., Brussels. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_003b.pdf

Sih, Andrew, and Robert D. Moore. "Delayed hatching of salamander eggs in response to enhanced larval predation risk." *American Naturalist* (1993): 947-960.

Sodhi, Navjot S., Bickford, David, Diesmos, Arvin C., Ming Lee, Tien, Koh, Lian Pin, Brook, Barry W., Sekercioglu, Cagan H., Corey, J.A., 2008 Measuring the meltdown: drivers of global amphibian extinction and decline. PLoS ONE 3(2), e1636. doi:10.1371/journal.pone.0001636.

Sorg, Barbara A., et al. "Exposure to repeated low-level formaldehyde in rats increases basal corticosterone levels and enhances the corticosterone response to subsequent formaldehyde." *Brain research* 898.2 (2001): 314-320.

Stuart, Simon N., et al. "Status and trends of amphibian declines and extinctions worldwide." *Science* 306.5702 (2004): 1783-1786.

Tollefsen, K.E., Salbu, B., Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO, and TiO2 to bacteria Vibrio fisheri and crustaceans Daphnia magna and Thamnocephalus platyurus. Chemosphere 71, 1308–1316.

United States Environmental Protection Agency, 1995. ZnO; Toxic Chemical Release Reporting; Community Right-ToKnow. Volume 60, Number 176

Wake, David B., Vrendenburg, Vance T., 2008. Colloquium paper: are we in the midst of the sixth mass extinction? A view from the world of amphibians. PNAS 105(Suppl. 1), 11466–11473. doi:10.1073/pnas.0801921105.

Warkentin, K. M. 1995. Adaptive plasticity in hatching age: a response to predation risk trade-offs. Proceedings of the National Academy of Sciences of the United States of America 92:3507–3510.

Warkentin, K. M. 1999. Effects of hatching age on development and hatchling morphology in the red-eyed treefrog, Agalychnis callidryas. Biological Journal of the Linnean Society 68:443–470.

Wingfield, J.C. (2005) The concept of allostasis: Coping with a capricious environment *Journal of Mammalogy,* **86,** 248-254.

Xiong, Daowen, et al. "Effects of nano-scale TiO 2, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage." *Science of the Total Environment* 409.8 (2011): 1444-1452.

Zhu, X et. al (2008). Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health. Part A, Toxic/hazardous Substances & Environmental Engineering*, *43*(3), 278–84.

Reflection

When I came to Utah State University, my interests were all over the board. I came to SOAR interested in forestry. I started Utah State University as a chemistry major because I was interested in science and the environment, but I had no experience in research at all. As a freshman, I was awarded the Utah State University Undergraduate Research Fellowship. As I began to look into different research programs, I realized chemistry was not for me. I was starting college, and I wanted to make the most of it. College was a place for me to start exploring what I enjoyed and wanted to work with as I moved forward. Because I needed to join a research lab due to my research fellowship, I started to look at different research programs. I have always enjoyed being outdoors and in the environment, so it is no surprise I found myself looking at the biology department. As I looked through the research different professors did in the biology department, I found myself interested in the physiology and ecology research that Dr. Susannah French was doing.

I began in Dr. Susannah French's lab the fall of my freshman year, and my four years since then have been a fantastic experience. Dr. French looks into how urbanization and habitat change affects organisms' physiology and health. I was interested in this lab because I grew up in Salt Lake City, and I saw the effects of urbanization first hand. I have been interested in how these types of habitat change, i.e. urbanization, habitat destruction, increased human influence, will affect ecosystems that are on the edge of these changes. Dr. French uses physiological measures, such as corticosterone, the immune system, and the reproduction system, to create a proxy to investigate how well an organism is doing. As I joined this lab, I thought that I would jump right into the

research process. I could ask a question and immediately start an experiment, but I learned right off the bat, in my first meeting with the lab actually, that this wasn't going to happen. I learned quickly that my idea of research was very misinformed.

When I started in the French lab, Dr. French was about to have a baby and one of the two graduate students, Lori Neuman-Lee, was about to take her comprehensive exams. Despite the lab being quite empty, I stayed in the lab because I was really interested in the subject. I began by taking care of laboratory animals with Marilize Van der Walt. We figured out what sex they are, fed the snakes, and made a better protocol for taking care of laboratory snakes working with the Savitzky lab.

Because I persisted in working and volunteering in this lab, I got a field technician job in the lab to catch lizards throughout the Arizona, Utah, and western Oregon. This job is what started my first independent research project. I collected blood samples and looked to see if the lizards had parasites in their blood. I analyzed if these parasites correlated with the physiological measures that the French lab usually measures. Because this was my first independent project, I began learning how to ask independent questions that were actually experiments and something you can answer. As I said earlier, I joined the lab thinking that I would start my own experiment right off the bat. After working in the lab for an over eight months, I began to learn everything that actually happened in the lab. Science is about asking new, well-specified questions that can actually be answered. I learned that the questions are not going to be large and vague, such as "How do parasites affect physiology?", but rather "How do endo- and ectoparasites affect corticosterone, the immune system, and testosterone?". The latter question, while not an encompassing and a "one-size fits" all answer to parasite biology,

is actually able to experiment on and answer. This is perhaps one of the most important things I have learned in my experience in my lab: how to take large and very interesting questions and parse them down into experiments that are both feasible and informational.

Following this, I began to be more independent in the lab. The following fall, I saw a poster at the biannual Biology Symposium put on by the biology department about the use of nanoparticles as a alternative to conventional pesticides. I asked the student how nanoparticles affect aquatic organisms that were likely to encounter them due to runoff or pollution, and he did not know. After a little bit of research, I found out that there was almost no research on aquatic organisms besides fish, and that there was none on salamanders. Because of this, I was interested in how nanoparticles would affect an animal we often use for experiments in my lab, the rough-skinned newt (a type of salamander).

For my original Honor's Thesis, I set up one experiment. I was going to examine how zinc oxide (ZnO) nanoparticles affected newt eggs. I began collecting eggs for my experiment, and when I collected enough, I realized the newts were still laying eggs. Because of this, I set up three more experiments, and I used almost 1600 newts in the process.

This was the first experiment that I was in charge of the entire research process. I came up with the idea, I applied for funding, I completed all of the data collection, I analyzed my results, and I am writing the manuscript. It was eye opening to see what this process entails. While I was studying biology, I learned about a lot of scientific material. I took physiology classes, ecology classes, taxonomic classes. I only ever took one English class, yet I spent more time writing my thesis and manuscript than I did coming

up with the idea or doing the data collection. I also was only required to take one statistics class for my major, but my mentor told me early to take as many statistics classes as possible. With this advice, I took four more stats classes, and I can already see how helpful it is.

While I was completing my thesis, I learned a lot about becoming a better scientist, writer, and student. I will be continuing my education at the University of Connecticut in the fall for a PhD program in ecology and evolution, and I will definitely use what I learned writing my thesis moving forward.

Biography

Austin Spence is graduating with a biology major with an emphasis in ecology and biodiversity and a chemistry minor. He also enjoys learning languages, and has taken two years of both German and French at Utah State University, leading to being German Student of the Year. Austin has worked in multiple ecology and genetic laboratories, both nationally and internationally, including several at Utah State University, Georgetown University in Washington DC, and the Senckenberg Museum of Natural History in Gelnhausen, Germany. Austin was a Goldwater Scholar Honorable Mention as well as a Utah State University Undergraduate Research Fellow. He will be continuing his education at a PhD program in ecology and evolution at the University of Connecticut in the fall of 2015.